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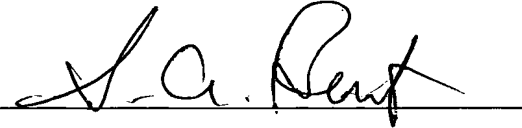
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It is respectfully requested that the references be placed in the patent file.

Respectfully submitted,

Date 20 October 2005

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NON PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.) date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ⁶
		KARWOWSKA et al., "Antibody levels to <i>candida albicans</i> carbohydrate and major cytoplasmic antigens isolated from standard and patient strains," Ann. Immunol. (Inst. Pasteur), 1984, 135D, pp. 145-159.	
		MAUCH et al., "Analysis of a solid-phase radioimmunoassay for antibodies to cytoplasmic antigen fractions of <i>candida albicans</i> ," Journal of Immunological Methods, vol. 43, 1981, pp. 181-192.	

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ANTIBODY LEVELS TO *CANDIDA ALBICANS* CARBOHYDRATE AND MAJOR CYTOPLASMIC ANTIGENS ISOLATED FROM STANDARD AND PATIENT STRAINS

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SUMMARY

A solid phase radioimmunoassay (RIA) and ELISA were used to detect human antibodies to *Candida albicans* (CA) organisms or purified fractions, namely, carbohydrate-rich fraction (CRF) and cytoplasmic peptides (SSF) of CA. IgG antibodies to either whole organisms or to CRF were found in sera obtained from patients with chronic mucocutaneous candidiasis (CMCC) or digestive candidiasis, as well as in healthy control sera. In patient sera, no correlation between the clinical stage of disease and the IgG anti-CRF levels was found. In contrast, antibodies to SSF were absent in healthy control sera. IgM anti-SSF in the absence of IgG anti-SSF were found in sera of patients with recent digestive candidiasis, and low levels of IgM and IgG Ab were detected in sera of CMCC patients.

The lack of correlation between IgG anti-CRF levels and clinical status can, in part, be explained by the individual variability of *Candida* strains and by the inadequacy of the laboratory standard antigens in the antibody assays used.

The clinical relevance of different tests to detect anti-CA antibodies is discussed.

KEY-WORDS: *Candida albicans*, IgM, IgG, Immunodepression, Candidiasis; RIA, ELISA, Human.

INTRODUCTION

Severe *Candida albicans* (CA) infections rank among the causes of mortality in immunocompromised patients [6]. The early diagnosis of deep-seated *Candida* infection remains difficult because the clinical presentation is usually non-specific and microbiological diagnosis is sometimes obtained only after invasive investigations. In this context, a meaningful serodiagnosis is needed. Several serological tests are used to measure the host immune response to CA, the antigen being either a homogenate of the whole microorganism or the cell wall mannan fraction. Such antigens are used for precipitation in agar [6], latex agglutination [19], counter-electrophoresis [1], radioimmunoassay (RIA) [13] and quantitative immunofluorescence [4]. Conflicting conclusions concerning the sensitivity and specificity of these techniques render the interpretation of results difficult.

These difficulties may be due to a number of reasons.

a) Antigen preparations and test methodology greatly differ. Most frequently, crude cell homogenates or cell-wall mannan fractions are used [1, 13, 14] and Axelsen *et al.* have identified as many as 78 antigenic components precipitated by human antisera [1]. To render detection of *Candida* antibody more reproducible through better definition of antigen, Jones [10] isolated and characterized a major cytoplasmic antigen of *Candida*. He found that the detection of antibodies against this antigen serves as a specific sign of severe *Candida* infection [10, 11].

b) Another reason which could explain some of the conflicting results in anti-*Candida* serology, may be the individual variability of antigen(s) [17, 18]. Individual antigenic differences could give rise to antibodies which would not interact with a laboratory standard strain antigen used for antibody detection.

The aims of this study were two-fold: 1) to obtain and characterize a carbohydrate fraction and major cytoplasmic CA antigen from either standard strains or strains isolated from patients; and 2) to compare antibody response toward these well-defined antigenic fractions using a solid phase RIA.

Sera from patients with chronic mucocutaneous candidiasis (CMCC) were used as a source of human antibodies. In contrast to systemic candidiasis, the diagnosis of CMCC has been rendered possible through

BCA = bovine serum albumin.
CA = *Candida albicans*.
CE = crude (cell) extract.
CMCC = chronic mucocutaneous candidiasis.
ConA = concanavalin A.
CRF = carbohydrate-rich fractions.

MW = molecular weight.
NHS = normal human serum.
PBS = phosphate-buffered saline.
RIA = radioimmunoassay.
SA = somatic antigen.
SPA = *Staphylococcus aureus* protein A.

the introduction of electrosinerosis [3]. Nevertheless, the antibody response towards the different *C. albicans* antigenic fractions should be qualitatively similar in chronic and systemic candidiasis.

MATERIALS AND METHODS

Culture of C. albicans.

C. albicans type A strain was obtained from Institut Pasteur (strain A *C. albicans* 3153).

Individual CA strains were isolated from the throats of two patients. Cultures were maintained on Sabouraud dextrose agar slants and expanded, when needed, in liquid Sabouraud medium at 37° C for 48 h.

Preparation and analysis of antigens.

For antigen preparation, organisms were collected by centrifugation in 0.15 M NaCl and killed by heating at 70° C for 1 h. After washing in phosphate-buffered saline (PBS), pH 7.1, whole cells were resuspended at 5×10^6 cells per ml and either used as antigen in radioimmunoassay (RIA), or further fractionated. Four antigenic fractions were prepared as follows.

1) Crude cell extract (CE): 10^7 of washed cells were resuspended in 10 mM phosphate buffer, pH 7.4, sonicated in ice (Sonic Power Soniquer, Branson Instruments) then centrifuged at 12,000 *g* for 15 min to remove particulate material and intact cells.

2) Somatic antigen (SA) was obtained from crude cell extract by centrifugation at 100,000 *g* for 60 min.

3) Major cytoplasmic antigen (SSF) was prepared according to Jones [7, 10] and passed through a ConA-Sepharose (Pharmacia Fine Chemicals) column to remove contaminating cell-wall mannan.

4) Carbohydrate-rich fraction (CRF) was prepared from crude cell extract by absorption on ConA-Sepharose which was pre-washed with 250 mM α -methylmannoside and then equilibrated with 0.50 mM-Tris-HCl buffer, pH 7.4, 150 mM-NaCl. Absorbed material was eluted with 250 mM α -methylmannoside. The eluate was exhaustively dialysed against PBS, and precipitated by ethanol. The precipitate was collected by centrifugation, washed twice with ethanol and vacuum dried.

In some experiments, commercially available somatic and metabolic CA antigens (Institut Pasteur) were used. Control antigens used were *Cryptococcus neoformans* obtained from the Institut Pasteur and *Toxoplasma gondii* purchased from the Institut Mérieux. Both antigens were used in RIA as a suspension of killed, washed cells. Tetanus toxoid was used at 20 μ g/ml. Protein content in SSF and CRF was measured by the method of Lowry [15] using BSA as a standard. Carbohydrates were measured by the cysteine reaction [12] using mannose as a standard.

Radiolabelling of CRF antigens was performed as follows. A tyramine-CRF conjugate was prepared as described by Jones *et al.* [9]. Tyramine-CRF was labelled with 125 I using the chloramine-T method [8]. Gel filtration analysis of iodinated CRF fractions was performed on an Ultragel AcA-22 column (90 \times 0.9 cm) in 10 mM Tris-HCl buffer pH 7.4, 150 mM NaCl, 20 mM EDTA. Polyacrylamide electrophoresis in the presence of SDS was performed according to Laemmli [14].

Radioimmunoassay (RIA) was performed using labelled SPA (protein A of

Staphylococcus aureus; Pharmacia Fine Chemicals) as the ligand. SPA was iodinated by the chloramine-T method to obtain a specific radioactivity of 10^7 cpm/ μ g. The assay consisted of five steps, all separated by 4 washings with PBS-Tween (0.01% w/v) to remove unbound reactants: 1) attachment of the antigen to solid phase-matrix; 2) incubation with PBS, 3% BSA, to saturate the solid-phase binding sites; 3) incubation with serum dilutions; 4) incubation with radio-labelled SPA; and 5) counting of the radioactivity bound to the solid phase. The following standard conditions were used. *C. albicans*, *C. neoformans* and *T. gondii* cell suspensions in PBS were used at 5×10^6 cell per ml. Purified specific and control antigen were used at 20 μ g/ml; 50 μ l aliquots per well were distributed in a multiwell dispose-tray (Linbro, Hamden, USA) and incubated for 2 h at room temperature. Incubation with dilutions of serum or antibody in binding or competitive assays lasted overnight at 4° C.

125 I-SPA, used at $200-400 \times 10^3$ cpm/well in PBS+3% BSA, was incubated for 2 h at room temperature.

ELISA.

Goat anti-human and IgM-alkaline-phosphatase-conjugated sera were purchased from Miles and used at 1/100 and 1/600 dilution. CA antigens (20, 50, or 100 μ g/ml) in sodium bicarbonate-carbonate buffer (10 mM, pH 9.5) were added to each well of a microtitre plate and then incubated with serum samples as for RIA. Fixed antibodies were detected by incubating with a suitable dilution of enzyme anti-Ig conjugates. Results are expressed in optical density units.

Anti-CA antibody from patient sera.

Sera drawn from patients with suspected or proven fungal infections and from normal blood donors were stored frozen at -20° C. Gastrointestinal candidiasis (GIC) was diagnosed histologically in tissue obtained from biopsy (serum TAR). Superficial mucocutaneous *Candida* infections were diagnosed by clinical appearance with confirmatory culture and stain of specimens from skin, oropharynx, vagina or urinary bladder (GRA, GAL, VAR). We also tested sera from patients without proven candidiasis but with anti-*Candida* serology (electrosinensis) suggestive of active infection (LES and BAB). Serum of patient YOL (CMCC) was not available.

RESULTS

Characterization of antigenic fractions.

Two SSF fractions were prepared, one from standard CA strain 3153 and another from individual strain LES. Their protein to carbohydrate ratios were, respectively, 1/0.12 and 1/0.028. Figure 1 depicts the polyacrylamide gel electrophoresis pattern of SSF fractions. A major peptide of apparent molecular weight 55 Kd was present in both SSF. SSF LES contained another peptide of MW 29 Kd.

FIG. 1. — Coomassie blue-stained SSF peptides prepared from the 3153 (a) and patient LES (b) CA strains and separated in SDS-PAGE (12.5%).

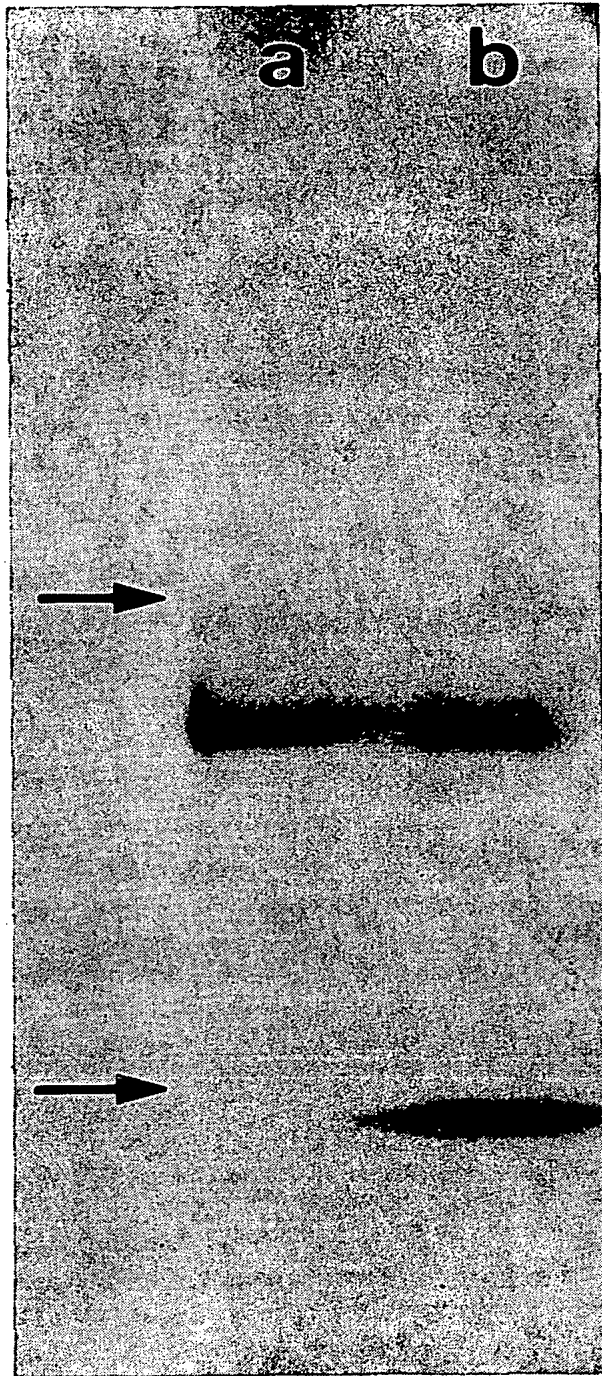


FIG. 1

Three carbohydrate-rich fractions obtained had minor protein contamination ranging from 0.2 to 0.6%. AcA-22 gel filtration profiles of iodinated CRF 3153, LES and YOL (fig. 2) showed a molecular size similarity. Both contained a high MW fraction (10^6) representing about 40% of total material, and heterogenous oligosaccharides (ranging from 6×10^5 to 10^4 MW) representing about 60%. The profile of CRF LES was somehow different; in this case, the 10^6 MW fraction represented only about 15% of total material, while the remaining fractions contained a 10^4 -MW molecule and some heavier species.

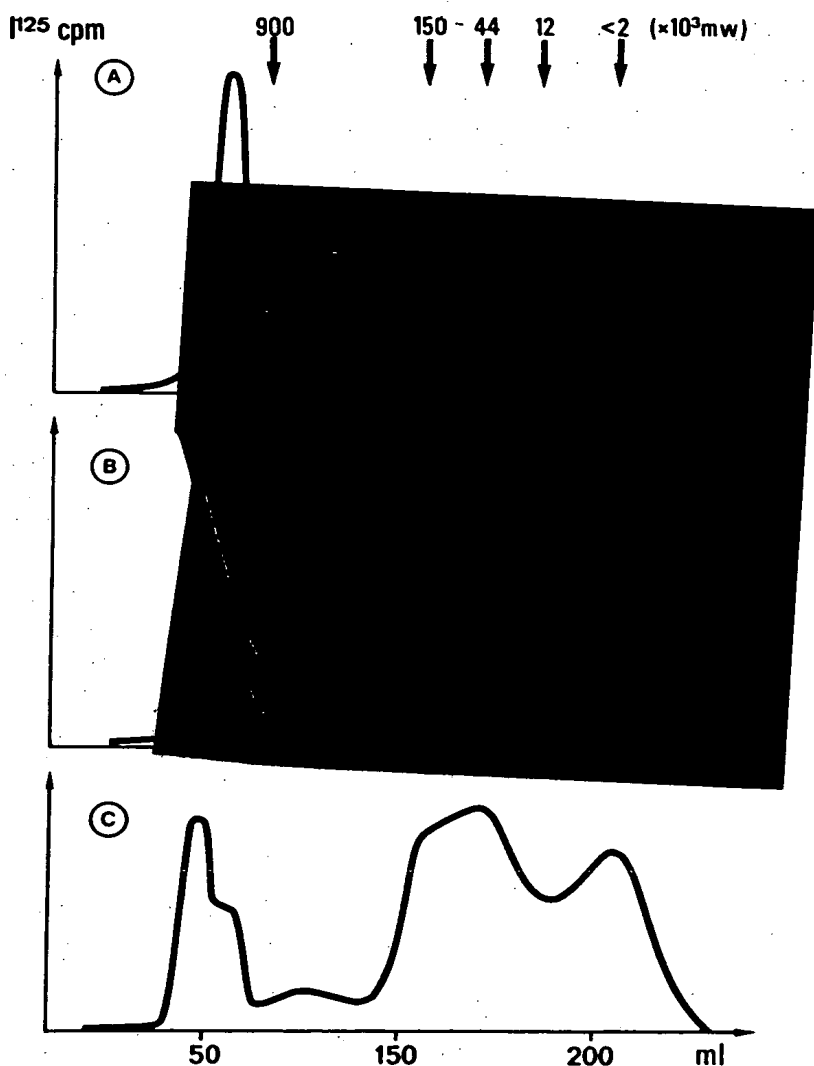


FIG. 2. — Gel filtration chromatography of iodinated CRF; 10^7 cpm of each CRF prepared from the 3153 (A), LES B and YOL (C) (CA) strains were added to an Ultrogel-AcA-22 column (90×0.9 cm) and eluted with 0.1 M Tris-HCl buffer.

Arrows indicate elution positions of molecular weight markers.

Detection of serum antibodies to C. albicans by RIA.

When whole CA organisms were used as a layer in RIA to detect antibodies in five anti-*Candida* and three control sera, variable ^{125}I -SPA binding was observed (table I). Sera from children with clinically evident chronic candidiasis (CBA, CAI) and serum from a patient with intestinal

TABLE I. — Detection of serum antibodies to whole « *Candida albicans* » by RIA.

	Serum dilution	¹²⁵ I-SPA binding (cpm)
BAB	PBS	1,010 (±226) (2)
	1/100	302,440 (±8,750)
	1/1,000	78,450 (±3,278)
LES	1/100	68,925 (±5,720)
	1/1,000	7,520 (±1,420)
GRA	1/20	36,300 (±2,960)
	1/100	4,290 (±1,020)
GAL	1/20	14,890 (±2,760)
	1/100	8,750 (±1,920)
TAR	1/20	9,612 (±1,680)
	1/100	1,050 (±1,020)
NHS1	1/20	72,400 (±6,990)
	1/100	31,300 (±4,100)
NHS2	1/20	19,650 (±2,980)
	1/100	7,800 (±1,350)
Pooled NHS	1/20	7,510 (±1,150)
	1/100	4,200 (±990)

Twenty-two other human sera from patients with suspected or proven *Candida* infections or from controls were tested. Since the dispersion of ¹²⁵I-SPA binding values was similar to that shown in this table, we give in this and the other tables data obtained for 6 patient sera, 2 controls and a pool of NHS.

Data represent the mean (±SEM) values of triplicate experiments performed with the same batch of ¹²⁵I-SPA for all sera tested except sera BAB and LES, which were assayed 22 and 18 times, respectively.

candidiasis (TAR) fixed more ¹²⁵I-SPA than the same dilution of pooled normal sera (36,300 cpm for serum GRA at 1/20, 14,800 and 9,612 cpm for sera GAL and TAR, respectively, as compared to 7,510 cpm for pooled NHS at 1/20). On the other hand, serum LES and BAB contained much more anti-*Candida* Ig, since, even at a dilution of 1/100, the ¹²⁵I-SPA binding values were 302,400 and 68,925 cpm, respectively. Finally, both sera from normal individuals (NHS1 and NHS2) displayed higher ligand binding values than either a pool of normal sera or three of four sera of CMMC and GIC when tested at 1/20 dilution.

The specificity of antibody was studied in binding and competitive assays. The binding of anti-*Candida* antibody to corresponding antigen was well inhibited by CA somatic antigen, completely inhibited by CRF fraction and negligibly inhibited by tetanus toxoid (table II).

Detection of antibodies to CRF and to major cytoplasmic antigen (SSF).

The binding of serum LES to different CRF and SSF antigen preparations was investigated (table III). The clearest binding is observed

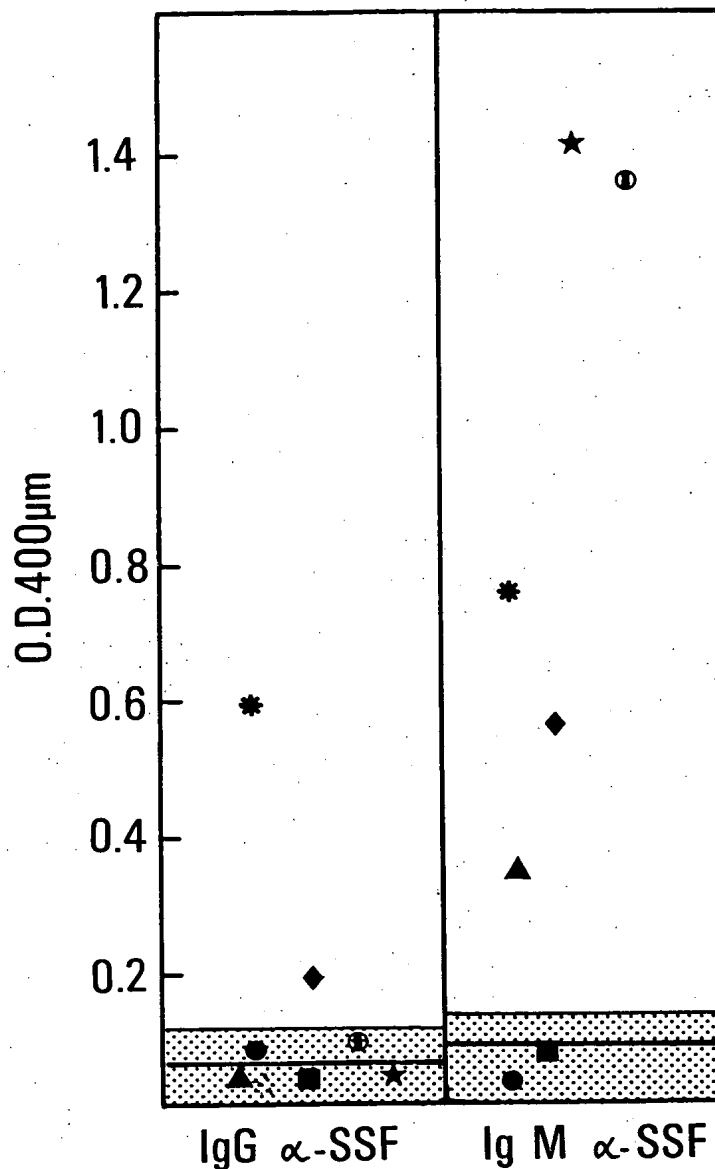


FIG. 3. — ELISA of IgG and IgM anti-SSF antibody binding in sera BAB (●), LES (*), GRA₇₈ (◐), GRA₈₀ (■), GAL (▲), TAR (★) and TEP (barred circle).

Serial dilutions of serum were incubated on SSF-layer (40 µg/ml). Results shown represent a mean of triplicate experiments of binding of 1/100 dilution of each serum, except for serum TAR where values of 1/1,000 dilution are shown. SE did not exceed 20% of the mean. Hatched series and solid line indicate the mean ± SEM of NHS binding.

to whole cell layer. Both CRF fractions gave similar binding, which was approximately 5 times lower than that observed on the cellular layer. The SSF fractions fixed very little antibody.

A poor binding of antibodies to SSF fractions was confirmed for all other sera tested, including those from patients with clinically evident *C. albicans* infection. As the SPA used as ligand would poorly detect antibodies of IgM class, we again tested these sera using ELISA. As shown in figure 3, control sera from normal individuals did not contain antibodies of either the IgG or IgM class against SSF. Out of 6 sera tested, serum LES contained IgM anti-SSF with a modest amount of IgG anti-SSF.

Three sera were negative. In contrast, sera from patients with recent severe digestive candidiasis (TAR, and TEP, a serum not tested in RIA) contained significant amounts of IgM anti-SSF in the absence of detectable anti-SSF IgG.

TABLE II. — Specificity of anti-« *Candida* » antibody binding on whole « *Candida albicans* » layer.

Serum (dilution)	Putative inhibitor (mg/ml)	¹²⁵ I-SPA binding (cpm)	Inhibition (%)
LES 1/200 ⁽²⁾	PBS	1,680 ± 160 ⁽¹⁾	—
	—	19,490 ± 2,630	—
	SA 3153 ⁽³⁾ 2.0	1,757 ± 450	91.0
	0.2	5,783 ± 1,230	70.2
	CRF 3153 ⁽³⁾ 0.5	1,840 ± 530	94.8
	0.05	1,942 ± 685	90.0
	Tetanus toxoid 2.0	16,875 ± 2,330	13.5
	0.2	17,870 ± 2,990	8.3

⁽¹⁾ Mean ± standard deviation of triplicate experiments performed with the same batch of ¹²⁵I-SPA.

⁽²⁾ Data obtained with 5 other CA sera were similar and are not shown.

⁽³⁾ Somatic antigen (SA) and CRF of *C. albicans* standard strain 3153.

TABLE III. — Binding of serum LES to « *C. albicans* » cells, CRF and major cytoplasmic antigen (SSF).

Ag layer	Serum tested (dilution)	¹²⁵ I-SPA binding (cpm)
CA 3153 5 × 10 ⁶ /ml	PBS	310 ± 160
	1/20	194,600 ± 11,370
	1/100	72,800 ± 8,100
CRF 3153 40 µg/ml	PBS	184 ± 95
	1/20	37,600 ± 4,260
	1/100	8,400 ± 1,580
CRF LES 40 µg/ml	PBS	204 ± 120
	1/20	38,280 ± 5,720
	1/100	7,060 ± 1,460
SSF 3153 50 µg/ml	PBS	591 ± 205
	1/20	4,720 ± 1,020
	1/100	2,280 ± 1,610
SSF LES 50 µg/ml	PBS	540 ± 320
	1/20	5,990 ± 1,130
	1/100	4,030 ± 1,020

Ag = antigen.

Mean ± SEM of triplicate experiments

TABLE IV. — Antigenicity of Ag fractions prepared from standard or autologous « *C. albicans* » strains.

Ag layer	Serum (dilution)	Putative inhibitor (200 µg/ml) ⁽¹⁾	¹²⁵ I-SPA binding (cpm)	Inhibition (%)
CE 3153 ⁽²⁾ 500 µg/ml	PBS BAB (1/1,000)	—	1,450 ± 230 ⁽⁴⁾	—
		—	19,000 ± 2,100	0
		CRF 3153	3,300 ± 580	82.5
		SA 3153 ⁽³⁾	2,040 ± 490	89.3
		CRF LES	11,500 ± 1,290	39.5
		SA LES ⁽³⁾	3,050 ± 630	84.0
	LES (1/1,000)	—	8,400 ± 990	—
		CRF 3153	1,600 ± 430	83.4
		SA 3153	2,047 ± 310	75.8
		CRF LES	4,895 ± 785	41.8
		SA LES	1,740 ± 390	79.2
	CE LES ⁽²⁾ 500 µg/ml	—	286,200 ± 15,330	—
		CRF 3153	3,280 ± 910	98.8
		SA 3153	3,980 ± 1,010	98.6
		CRF LES	54,230 ± 4,780	79.8
		SA LES	2,470 ± 660	99.1
	LES (1/1,000)	—	78,000 ± 8,760	—
		CRF 3153	1,500 ± 460	97.9
		SA 3153	2,712 ± 980	96.6
		CRF LES	6,850 ± 1,210	92.3
		SA LES	2,380 ± 930	97.0

⁽¹⁾ µg of carbohydrate.⁽²⁾ Crude cell extracts prepared from both 3153 and LES strains.⁽³⁾ Corresponding somatic antigen.⁽⁴⁾ Mean ± SEM of triplicate experiments performed with the same batch of ¹²⁵I-SPA.

Detection of anti-CA antibodies in RIA using the CA strain 3153 antigens or CA antigens prepared from one patient's CA strain.

To compare the antigenicity of the individual and standard CA strains, their CRF and somatic antigens were used as inhibitors of antibody binding (table IV). The somatic antigen from LES CA strain gave similar inhibition values to those from strain 3153. However, the CRF fraction from LES inhibited by only 40% the fixation of LES and BAB antibodies on a 3153 layer. An almost complete inhibition was obtained when the same antibodies were fixed on autologous LES CA layer.

DISCUSSION

We have isolated and partially characterized carbohydrate cell wall antigens and a major cytoplasmic antigen from two individual *C. albicans* strains. These antigens have been compared with similar antigenic fractions from the standard *C. albicans* strain 3153. All CRF fractions were

found to be heterogeneous in molecular size distribution. CRF LES differed from standard and other individual CRF in that it contained a significant proportion of small molecular size molecules. Both SSF preparations contained a peptide of apparent MW of 55 Kd, which appears to be similar in MW to a major cytoplasmic antigen isolated by Jones [9]. The 29 Kd peptide is probably a degradation product, since its quantity increased after storage.

The solid phase RIA we developed was used to measure the level of antibody against CA and to study the antigenicity of isolated CA fractions. Firstly, we confirmed that anti-*Candida* antibody levels are indeed difficult to correlate with clinical manifestations of the disease. Relatively low antibody titres were observed in sera GRA and GAL taken from patients during severe mucocutaneous and digestive invasion of *Candida*. Moreover, sera from two non-infected individuals contained significantly more IgG antibodies than the MCC sera.

When the CRF fractions were used as the antigen layer, sera rich in IgG anti-*Candida* cells were found, without exception, to be positive in IgG anti-CRF, although binding observed was lower than on the whole-cell layer. This is probably due to the larger antigenic surface available to antibodies when the cells are used. Since the CRF fractions were all found to behave as inhibitors of antibody binding to the cellular layer, both binding assays therefore detect the same antibody populations, directed mainly to carbohydrate Ag of CA.

When the SSF was used as antigen, no antibodies against this fraction were detected in normal human sera. This result is in agreement with the observations of Jones [11] and contrasts with the ubiquitous presence of antibodies to cell wall antigens. The only sera having a high titre of IgM anti-SSF in the absence of IgG anti-SSF were obtained from patients with recent severe intestinal candidiasis. Two out of four CMCC sera with moderate IgG anti-CRF levels had low titres of IgG and IgM antibodies to SSF; two others were found to be negative. Taken together, these observations show that the appearance of serum IgM antibody to SSF correlates with recent *Candida* infection.

Some differences in gel filtration profiles were detected between LES and standard 3153 CRF fractions. Also, CRF failed to completely inhibit antibody binding to standard Ag layer, while this was not the case when antibodies were bound to autologous antigen. This may reflect the individual phenotypic variability of parietal antigens of *C. albicans* described by Poulain *et al.* [18]. Such variability may explain the difficulties in detecting Ab populations raised against newly formed determinants.

The detection of anti-*Candida* cell wall (or CRF) antibodies in normal sera and their high levels in some individuals without clinical symptoms of candidiasis raises the question of the clinical relevance of this type of antibody assay. The major cell wall antigen of *C. albicans* is mannan [20]. In contrast with mice [11], humans respond easily to this antigen and produce quite significant amounts of anti-mannan IgG [11, 15]. Mannan, like all carbohydrates, is catabolised slowly

and can be detected in patient sera with systemic and chronic candidiasis. Specific immune complexes may play a role in the control of antibody response, and we were able to demonstrate that they also modify the serological tests used to detect anti-*C. albicans* antibodies (Lisowska-Groszpiere *et al.*, manuscript in preparation). The rationale of any serodiagnosis of *Candida* is that only infection, as opposed to non-pathogenic « colonization », should stimulate detectable antibody response. If the pre-existing level of antibody is high, an increase in titre may not always be detectable or meaningful.

The use of a solid phase immunoassay does not solve these problems. On the contrary, it renders them even more acute due to the higher sensitivity of this assay. Our observations indicate that the use of a relevant antigen preparation is a critical factor.

RÉSUMÉ

ANTICORPS ANTI-« *CANDIDA ALBICANS* »
DIRIGÉS CONTRE LES ANTIGÈNES POLYOSIDIQUES
ET CONTRE LES ANTIGÈNES CYTOPLASMIQUES MAJEURS ISOLÉS
D'UNE SOUCHE STANDARD OU DE SOUCHES RETROUVÉES CHEZ LES MALADES

Des dosages radioimmunologiques (RIA) et/ou enzymoimmunologiques (ELISA) ont été utilisés pour détecter les anticorps humains dirigés contre *Candida albicans* et contre ses fractions antigéniques : fraction riche en hydrates de carbone (CRF) et peptides cytoplasmiques (SSF). Les anticorps de classe IgG anti-*C. albicans* et anti-CRF ont été détectés dans les sérums des malades atteints de candidose cutanée muqueuse chronique (CCMC) ou digestive, et dans tous les sérums témoins. Aucune corrélation n'a été établie entre les taux de ces anticorps et l'évolution clinique de la maladie. Par contre, les anticorps anti-SSF de *C. albicans* n'ont été détectés que dans les sérums de sujets atteints de candidose : il s'agit d'anticorps à des taux faibles de classe IgG et IgM chez les malades atteints de CCMC.

L'absence de corrélation entre les titres d'IgG anti-CRF et l'évolution de la maladie peut être expliquée, en partie, par une variabilité individuelle des souches de *C. albicans*. La signification clinique des différents tests de détection des anticorps anti-*C. albicans* est discutée.

MOTS-CLÉS : *Candida albicans*, IgG, IgM, Immunodépression, Candidose; RIA, ELISA, Homme.

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ANALYSIS OF A SOLID-PHASE RADIOIMMUNOASSAY FOR ANTIBODIES TO CYTOPLASMIC ANTIGEN FRACTIONS OF *CANDIDA ALBICANS*

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An indirect solid-phase radioimmunoassay (SPRIA) in individual polystyrene micro-titer cups has been adapted for measurement of antibody to various cytoplasmic and carbohydrate antigen fractions of *Candida albicans*. The assay was optimized for sensitivity, precision and linearization of serum dilution curves. The optimized procedure allows computerized measurement of anti-*Candida* antibodies and can be used for measurement of antibody over a wide concentration range. The procedure obviates variation due to changes in day-to-day counts as a result of isotope decay and end-point antibody dilutions. The assay has been used to demonstrate a Poisson-like distribution of antibody levels in the sera of persons showing no symptoms of candidiasis. The minimum antibody level detectable by the assay is about two orders of magnitude lower than the lowest level found in human serum and 4 orders of magnitude lower than the most sensitive test used hitherto, the hemagglutination test.

INTRODUCTION

Serodiagnosis in invasive candidiasis requires distinctions to be made between simple superficial colonization by the *Candida* species, infection of the mucous membranes or skin, and serious deep-tissue infection which may lead to septicemia. Different immunological techniques including double diffusion precipitation, passive hemagglutination and crossed immunoelectrophoresis have been used to detect antibodies to *Candida albicans*. However, the findings are controversial and their interpretation is difficult (Axelsen, 1976; Glew et al., 1978; Kozinn et al., 1978).

The antigen spectrum of *C. albicans* is complex. Besides the cell-wall (mannan) antigen there are at least 78 cytoplasmic (protein) antigens (Axelsen, 1976) which stimulate antibody production. Taschdjian et al. (1969) postulate that exposure of cytoplasmic antigens should not occur unless the *Candida* cells invade tissue. Axelsen (1976) found antibody to some of the cytoplasmic protein antigens to be specific for deep-seated *Candida* infection. Thus detection of antibodies to the various cytoplasmic antigens should aid the serological diagnosis of possibly fatal invasive candidiasis. A rise in specific antibody concentration was considered to be of diagnostic

value in deep tissue infections by *Candida albicans* (Glew et al., 1978; Kozinn et al., 1978). The significance of a two-fold or three-fold rise in titer as determined by conventional tests is doubtful since it may be within the range of error of such tests (Kozinn et al., 1978).

Recently, radioimmunoassay with killed yeast cells as antigen have been used by Tripatzis (1978), Cobb and Parratt (1978), and Szöllösy et al. (1979) to detect antibodies to cell-wall components. We describe here a modification of the micro-solid-phase radioimmunoassay (SPRIA) (Rosenthal et al., 1972), which allows sensitive and quantitative detection of antibodies against cytoplasmic antigen fractions. The aim of this study was also to optimize the SPRIA conditions for parallel use of various separated antigens. Antibodies to both whole cytoplasmic antigen and isolated carbohydrate antigen fractions of the cytoplasmic extract were measured.

MATERIALS AND METHODS

Solid-phase support and preparations of antigens (Q)

The antigen was immobilized by attachment to the internal wall of individual polystyrene microtiter cups embedded in styropore (Removawell system, Dynatech, Nürtingen, F.R.G.). Various types of cups were tested: M 74 A (U-shaped cups), M 174 A (Immunolon cups), M 179 A (F-cups).

A crude cytoplasmic extract (Q_a) was obtained by homogenization of yeast-phase cells of *C. albicans* (strain DSM 70010, Göttingen, F.R.G.) grown in Sabouraud-dextrose broth (Difco) for 48 h at 30°C. The cells were collected by centrifugation ($1000 \times g$, 10 min), washed 3 times with 0.9% NaCl and disrupted in a Braun desintegrator (Fa. Braun, Melsungen, F.R.G.) with 1 mm glass beads. Cell debris was removed by ultracentrifugation ($100,000 \times g$, 120 min, 4°C). The supernatant containing cytoplasmic material was concentrated and subjected to phenol-water extraction (Müller et al., 1964) to yield the 'carbohydrate fraction' (Q_b). This antigen is used also in a commercial hemagglutination assay and was kindly made available by Prof. H.-L. Müller (Hoffmann-La Roche, Basel, Switzerland). Further antigen fractions were purified from the cytoplasmic fraction by affinity chromatography on Sepharose-concanavalin A (Pharmacia, Uppsala, Sweden) (Longbottom et al., 1976). The first wash peak, containing mannan-free protein, was concentrated by negative pressure dialysis in collodium bags and is referred to as 'purified cytoplasmic antigen' (Q_c). The material retained by the concanavalin A was eluted with 1,0-methyl- α -D-mannopyranoside (Sigma, Munich, F.R.G.). This fraction contained polysaccharides and was termed 'purified carbohydrate antigen' (Q_d). The antigens were diluted in the buffer described by Teorell and Stenhagen (1938) at pH values suitable for optimal attachment to the polystyrene surface.

Protein concentrations were determined according to Bradford (1976).

Anti-Candida antibody (P_1)

Sera from 50 healthy adults and 50 hospitalized adults without evidence of *Candida* infection were tested, and also from 50 symptom-free children of various ages. Only 2 of hundreds of tested sera from patients were included in this study. The sera were kept frozen at -20°C . Serum dilutions were in phosphate buffer, 0.1 mol/l, pH 7.2 (PB), containing 0.2% bovine serum albumin (PB-A).

*Preparation of radiolabelled antibody (P^*_2)*

Rabbit anti-human IgG was prepared as described previously (Mauch and Kümel, 1979a) and purified on a human IgG-Sepharose column (Mauch and Kümel, 1979b). The antibody was checked for specificity by immunoelectrophoresis according to Scheidegger (1955). Iodination with ^{125}I (Amersham, Braunschweig, F.R.G.) was by a modification of the chloramine-T method (Mauch and Kümel, 1979a). Briefly, 1 mCi of ^{125}I and 50 μg chloramine-T were added to 2.5 mg of the purified anti-human-Ig antibody in 4 ml PB. After 5 min the reaction was stopped by adding 50 μg sodium metabisulfite. Free iodine was removed by extensive dialysis against PB. The specific radioactivity was 0.50 $\mu\text{Ci } ^{125}\text{I}/\mu\text{g}$ protein.

Test procedure

The assay consisted of 7 steps: (1) attachment of *Candida* antigen to the solid phase matrix; (2) pre-wash (to remove unabsorbed antigen); (3) first incubation with standard or unknown anti-*Candida* antibody (P_1) (formation of antigen-antibody complexes QP_1); (4) first wash (to remove unbound anti-*Candida* antibodies); (5) second incubation with radiolabelled anti-human-Ig antibody (P^*_2) with formation of complexes (QP_1P^*_2); (6) second wash (to remove unbound radiolabelled antibodies); (7) counting of the radioactivity (B) bound to the solid phase.

The following standard conditions were used: The cytoplasmic protein antigen (Q_a) in Teorell buffer, pH 5 (50 μg) and the carbohydrate fraction (Q_b) in Teorell buffer, pH 9 (8 μg) were air-dried in the cups at room temperature, from a volume of 50 μl . The first and second incubation times were both 1 h. All incubations were at 37°C . 0.15 mol/l phosphate-buffered saline (PBS), pH 7.2, was used as wash buffer. The cups were counted in a gamma-ray spectrometer (Kontron MR 252) and the antibody concentration was computed by a programmable desk-top calculator (Hewlett Packard, HP 9815 A).

Measurement of antibody levels

The radioactivity measured defines the amount of radiolabelled antibody bound to the cup. Experiments with heterologous antibody P_1 showed non-specific binding to be negligible. Thus the total amount of radiolabelled antibody P^*_2 present was immunospecific complex QP_1P^*_2 . Since the specific activity of the radiolabelled antibody decays, the binding ratio B/B_0 (where

B_0 is the total radioactivity added) was used as response variable, so that identical dose-response curves were obtained for different values of specific radioactivity. The assay gave non-linear dose-response curves when the binding ratio was plotted against the antibody concentration p_1 ('dose' of antibody) or against $\log p_1$.

The concentration of anti-*Candida* antibodies (p_1) was 0.25 MAN (MAN = multiple of adult norm (see Results)), 1 MAN being equivalent to 20 μg antibody/ml, approximately. The concentration of radiolabelled second antibody (p_2) was 0.2 $\mu\text{g}/50 \mu\text{l}$ ($B_0 = 100,000$ cpm).

The conditions of the assay to obtain the required sensitivity (minimum detectable concentration and concentration difference) and precision (repeatability) were empirically optimized by varying the factors influencing assay slope and variance in the response variable and the interpolated antibody concentration.

RESULTS

Attachment of antigen to the solid-phase support

The antigen fractions Q_a and Q_b are mixtures of proteins and carbohydrates. Their adsorption characteristics depend on the solid-phase support chosen and on the buffer, time, temperature, and antigen concentration.

Solid phase. The antigens used adhere to the wall of the microtiter cup. The volume (v) was 50 μl and the exposed surface area 50 mm^2 . Fig. 1 shows the influence of the type of cup on the dose-response curve for 'cytoplasmic protein' antigen Q_a and carbohydrate antigen Q_b . With M 74 A cups, slightly better results were obtained than with the other cups, and so these were used in further experiments.

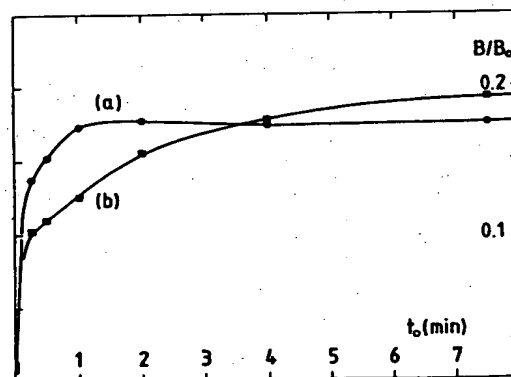
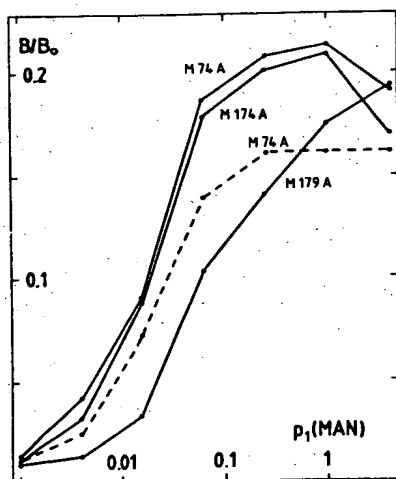


Fig. 1. Effect of type microtiter cup used on dose-response. Solid lines: antigen Q_b . Dashed line: antigen Q_a . See text for explanation of MAN.

Fig. 2. Antigen binding as a function of time, t_0 , at 4°C. a: cytoplasmic antigen Q_a . b: carbohydrate antigen Q_b .

Buffer. Antigen Q_a contained mainly protein antigens and its dose-response curve strongly depended on the buffer used. The binding ratio B/B_0 slightly increased (+20%), if the pH of the Teorell buffer changed from 7 to 2 (data not shown). Use of distilled water instead of buffer reduced the binding to 50%. Teorell buffer, pH 5, was chosen as standard for Q_a . In contrast to the adsorption of Q_a , the use of distilled water or change of pH had no substantial effect on the adsorption of the carbohydrate antigen Q_b .

Time, temperature and antigen concentration. Representation time curves for the adsorption process are shown in Fig. 2. The surface saturation by antigen appeared to be completed for both Q_a and Q_b after a few minutes. The effect of a further increase of adsorption time appeared to be complex. For both Q_a (Fig. 3a) and Q_b (Fig. 3b) at least two phases of adsorption were seen, presumably as a result of competition for polystyrene binding sites between various antigen components and contaminants of the complex crude antigen fraction. As the area density of adsorbed components increased, the affinity for further binding might differ for each, with different kinetics. If the SPRIA assay with standard serum and under standard conditions was used to assess adsorption, the binding ratio B/B_0 increased rapidly at the beginning of the adsorption process, reached a plateau and decreased. After a long time interval the binding ratio increased again and reached a final plateau after 5 h. The first B/B_0 increase did not occur reproducibly at the same time after the start of adsorption. For adsorption times of less than 4 h, the composition of the adsorbed antigens and hence the composition of the complementary antibodies, varied in several assays performed after equal adsorption times. Thus the interassay variation of the interpolated antibody concentration measured in the SPRIA was high (up to 50%). To limit the interassay variation, an adsorption time longer than 4 h was used. Adsorption at 4°C was slightly better than adsorption at 22°C and 37°C.

In Fig. 4, dilution curves of the carbohydrate antigen (Q_b) obtained with varying incubation times are shown. Drying of the antigen to the polystyrene surface (2 days at 22°C) instead of adsorption (18 h at 4°C) slightly improved sensitivity and interassay variation. Air-drying at room temperature was therefore adopted as a standard procedure. Maximum sensitivity was reached for antigen concentrations of 1 $\mu\text{g}/50 \mu\text{l}$ reaction volume. Cytoplasmic antigen Q_a , although more complex than carbohydrate antigen Q_b , gave similar curves.

Washing and non-specific binding

The effect of varying the incubation time with the washing buffer on the dose-response curve was small. The washing procedure was repeated. Varying concentrations of Tween 80 or Tween 20 in the wash solution did not improve the assay.

Binding of the radiolabelled antibody to unsaturated binding sites of polystyrene and/or antigen led to background radioactivity ($B/B_0 = 0.005$, approximately). Coated cups containing PB-A instead of antiserum were

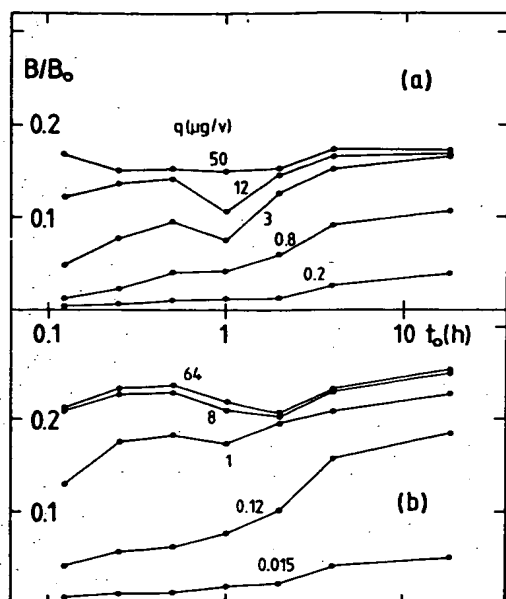


Fig. 3. Antigen binding as a function of time, t_0 , at 4°C. Antigen concentration is the parameter (reaction volume, $v = 50 \mu\text{l}$). a: cytoplasmic antigen Q_a . b: carbohydrate antigen Q_b .

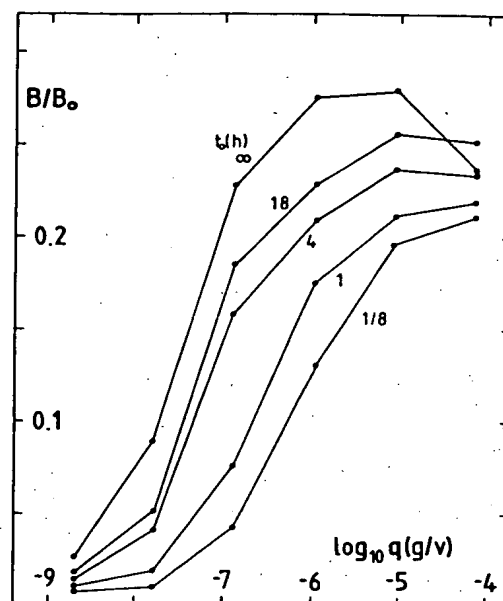


Fig. 4. Effect of incubation time, t_0 , of carbohydrate antigen, Q_b , on antigen dilution curves at 4°C (reaction volume, $v = 50 \mu\text{l}$). q = concentration of antigen; t_0 (h) = ∞ indicates air-dried antigen.

used as background controls. The effect of 2% w/v bovine serum albumin in PB in blocking remaining binding sites after antigen attachment was tested (incubation for 4–18 h at 4–37°C). This gave no reduction of non-specific binding with pH 5-buffered cytoplasmic antigen Q_a , air-dried onto the polystyrene. Blocking with albumin was, therefore, not adopted.

First and second incubations

After incubation of appropriately diluted human serum containing anti-*Candida* antibodies, the antigen-antibody reaction reached equilibrium very quickly (Fig. 5a). Incubation for longer than 1 h did not give greater binding rates. Stepwise variation of the incubation temperature from 4 to 37°C, increased the high-dose hook effect (Fig. 8a).

The second radiolabelled antibody (P_2^+) was used at appropriate dilution (p_2) and allowed to react with the antigen-antibody complex. Dilution curves for the radiolabelled antibody P_2^+ with p_1 as parameter are shown in Fig. 6b. With increasing concentrations of P_2^+ ($=p_2$), the measured activity B increased, but the binding ratio B/B_0 decreased, since the bound radioactivity (B) did not increase proportionally with the concentration of P_2^+ . The maximum assay slope was measured at $p_2 = 0.2 \mu\text{g}/50 \mu\text{l}$ ($B_0 = 10^5$ cpm), i.e. maximum discrimination of the binding ratios was achieved between $p_1 = 0.1$ and 0.004 MAN (Fig. 6b). Increasing the concentration of radiolabelled

antibody p_2 (from 0.2 to 0.6 $\mu\text{g}/50\ \mu\text{l}$, Fig. 6a) lowered the binding ratio and assay sensitivity and gave a more pronounced high-dose hook effect.

Figs. 5 and 7 show the effect of varying the incubation time. Although for P_2^+ a greater binding ratio was obtained with incubation times as long as 8 h (Fig. 7; Fig. 5, curve b), satisfactory discrimination was observed for p_1 between 0.004 and 0.6 MAN (Fig. 7) after 1 h. The optimum temperature was 37°C (Fig. 8b).

Routine assay procedure and quantitation of antibody levels

The cups were coated with antigen (50 μl) by air-drying for 2 days at room temperature as described. After duplicate washing with 100 μl PBS at room temperature, the coated cups were stored at 4°C until use. Fifty μl of diluted human serum was incubated for 1 h at 37°C followed by a washing step and addition of 0.2 $\mu\text{g}/50\ \mu\text{l}$ radiolabelled antibody for 1 h at 37°C. Two cups for each serum dilution and each antigen were used in the assay. Detection of antibodies against antigens (Q_a and Q_b) was performed at the same time.

Fig. 9 shows titration curves of three sera, containing low, medium and high levels of anti-*Candida* antibody, respectively. Serial dilutions in \log_2 steps were prepared for each serum. The abrupt decline of the titration curve in the range of moderately diluted serum corresponds to the descending branch in the dose-response curves (high-dose hook effect). With a pool of normal sera (mean value = 1 MAN) as standard (standard concentration = c_s), the relative antibody level of an unknown serum (c) is given by

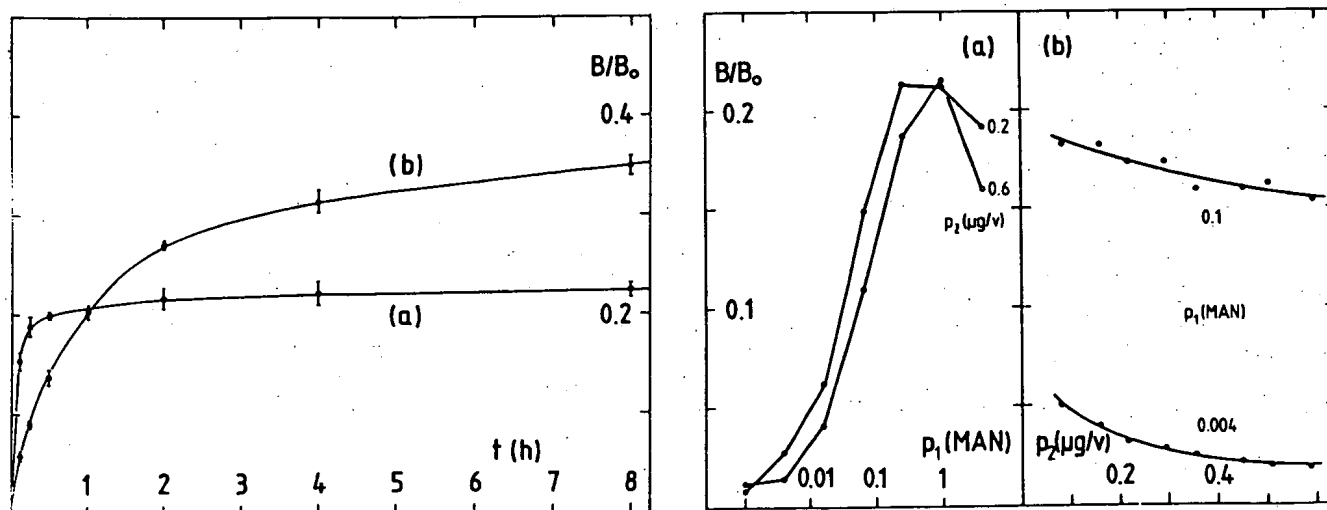


Fig. 5. Effect on binding ratio of varying the incubation time t_1 (curve a) of anti-*Candida* antibody and t_2 (curve b) of radiolabelled second antibody.

Fig. 6. a: effect of varying the concentration of the second labelled antibody (p_2) on the dose-response curve. b: binding ratio as a function of concentration of labelled antibody (p_2). Concentration of anti-*Candida* antibody p_1 (expressed in MAN = multiple of adult normal serum) = 0.1 MAN. Reaction volume = 50 μl .

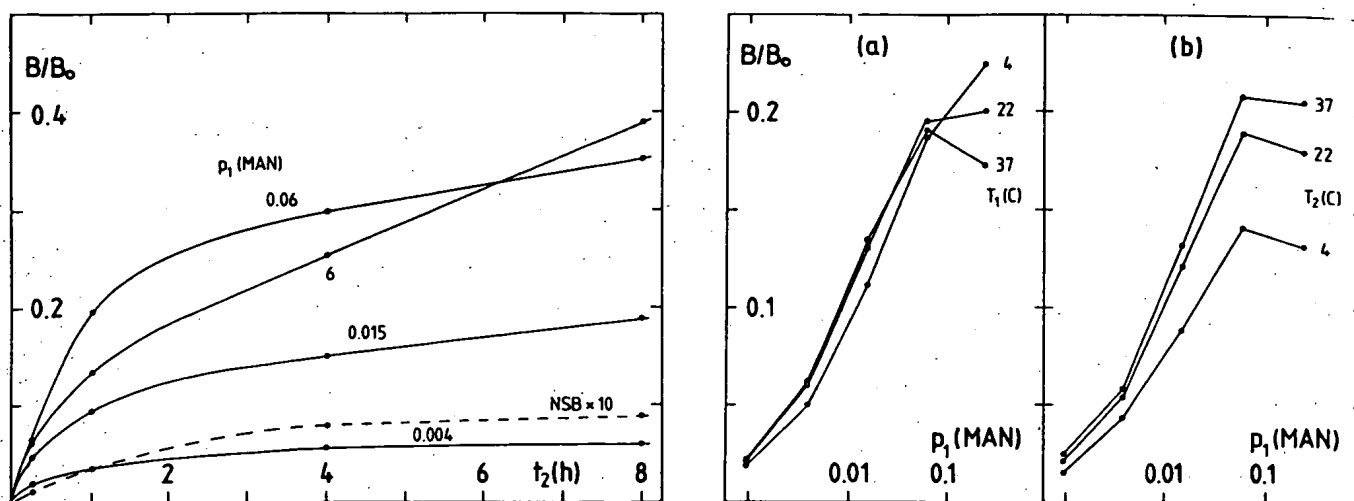


Fig. 7. Effect on the binding ratio of varying the incubation time t_2 of the radiolabelled antibody. Anti-*Candida* antibody concentration p_1 (0.004–6 MAN) is the parameter. NSB = non-specific binding.

Fig. 8. Effect of varying the incubation temperature T_1 (a) for the first antibody and T_2 (b) for the radiolabelled second antibody on the dose-response curve. The abscissa represents the dilution of anti-*Candida* antibody containing serum. (p_1 = concentration of anti-*Candida* antibody.)

$c/c_s = 2^d$, where d is the distance between the associated curves (in units of \log_2) for a mean response level optimized for precision.

Owing to the hook effect, all samples in the assay need to be analyzed at two or more concentrations to ascertain whether the measured value marks the ascending or descending part of the curve. Four standard dilutions of each serum ($D = 1 : 4, 1 : 16, 1 : 64, 1 : 256$) proved sufficient for computerized analysis. The intraassay variation inherent in this method was less than 15%, if an automatic washer was used (Dynawasher, Dynatech, Plochingen, F.R.G.).

Distributions of antibody levels

The distribution of antibody level for normal sera was evaluated. One hundred sera from candidiasis-free adults were tested. Fig. 10 shows the distribution of antibody levels against Q_a and Q_b , respectively. The mean value is normalized to one, so that all antibody levels are expressed in multiples of adult norm (MAN) with regard to the antigen fraction used. This unit of antibody concentration has immediate clinical relevance and has been used throughout this paper. 1 MAN is equivalent to approximately 20 $\mu\text{g/ml}$ anti- Q_a antibody. The minimum concentration detectable in the assay is 0.0004 MAN. All normal sera contained concentrations above 0.05 MAN.

The use of 'purified' cytoplasmic antigen (Q_c) produced a sharp decrease in sensitivity; hence it was not possible to establish the range of antibody levels for normal sera. Surprisingly, with this substance as test antigen no anti- Q_c antibodies could be detected in the sera of patients with *Candida* septicemia who had high levels of antibodies against cytoplasmic antigen Q_c .

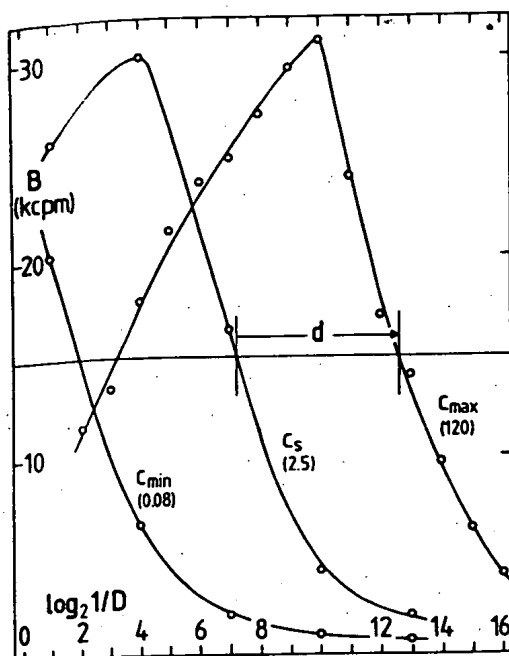


Fig. 9. Bound activity B (in 10^3 cpm) as a function of reciprocal serum dilution. Sera with minimum (c_{\min}), standard (c_s) and maximum (c_{\max}) concentration of anti-*Candida* antibody (values in MAN) are diluted in steps of \log_2 . Distance d (in units of \log_2) between the titration curves of unknown serum (with concentration c) and standard serum at an appropriate level of B is related to the concentration ratio by $c/c_s = 2^d$.

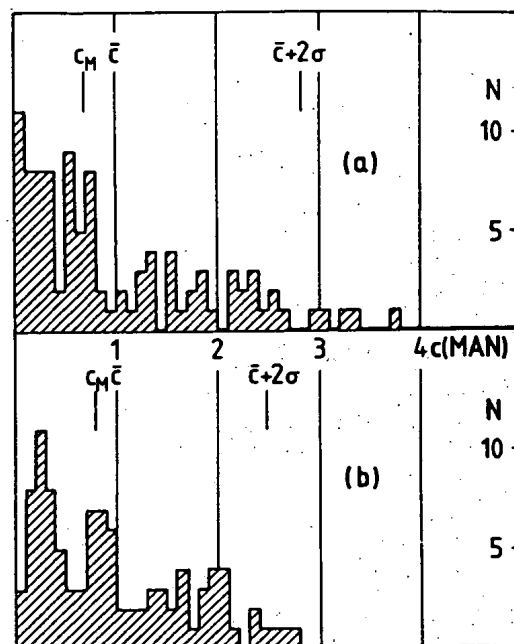


Fig. 10. Normalized frequency distribution of concentration c . a: antibody to whole cytoplasmic antigen Q_a . b: antibody to cytoplasmic carbohydrate antigen Q_b in sera of candidiasis-free adults. N = number of sera; mean value $\bar{c} = 1$ MAN; median c_M , 95%-confidence limit $\bar{c} + 2\sigma$.

whereas large amounts of anti- Q_c antibodies were found in sera from patients with superficial candidiasis (vaginal, esophageal), which in turn showed low levels of anti- Q_a antibodies. At present, there is no apparent explanation for these findings.

With reference to the distribution of antibody levels in normal sera the purified carbohydrate antigen (Q_d) was comparable with the test antigen Q_b .

DISCUSSION

The assay consists of two sequential reactions (formation of QP_1 and $QP_1P_2^+$) which depend on the law of mass action. From mass-action equations, an S-shaped dose-response curve is obtained (assuming a low affinity constant for the first reaction $K_2 = 1$, and an intermediate affinity for the second reaction, $K_2 = 5$), which fits the low-dose region of an empirical curve. However, the presence of side reactions due to heterogeneity of Q , P_1 and P_2^+ affects the shape of the assay curve (Rodbard and Feldman, 1978). The high-dose hook effect has likewise been observed in the solid phase enzyme-linked immunosorbent assay (Bruins et al., 1978) and the two-site immunoradiometric (sandwich) assay (Miles, 1977). Bruins et al. (1978) me that low affinity antibodies are unable to resist washing when they

are crowded and poorly aligned on the antigenic site. In the SPRIA, Zolinger et al. (1976) found that this effect was caused by an excess of antigenic sites on the coated surface. We conclude from our findings, that the high-dose hook is influenced by many parameters, and should be eliminated for automated (computerized) analysis. The high-dose hook effect is explained by antibody heterogeneity and/or incomplete washing (Rodbard and Feldman, 1978).

Assuming a mixture of two antibodies of different affinity, Rodbard et al. (1978) predicted the high-dose decline in the sandwich assay. At high-dose levels, a large amount of low-affinity complexes QP_1 is formed, and these dissociate rapidly during the second incubation. Thus free first antibody P_1 is released which can react with radiolabelled second antibody P_2^+ , lowering the amount of P_2^+ available in the reaction with immobilized QP_1 . The hook effect should thus be enhanced with increasing incubation time, t_2 . Fig. 7 shows that the binding rate at a high-dose level ($p_1 = 6$ MAN) is lowered with an intermediate incubation time for radiolabelled antibodies (t_2), but increases markedly with further increase of t_2 . Increasing t_2 up to several hours results in reduction of the high-dose hook effect. Unbound anti-*Candida* antibody P_1 appearing during the second incubation might have been due to incomplete washing. We found that prolonging the first washing time (15 min instead of 5 min) gave only a slight reduction of, but did not suppress, the hook. On the other hand, rapid suction after the first incubation enhanced the hook effect. We have been unable to reproduce the effect quantitatively or to eliminate it at high antigen concentrations (50 μ g protein/v and 8 μ g polysaccharide/v). This restricts use of the dose-response curve at the intermediate and low-dose segments. In these regions, the transformed dose-response curve (with log binding ratio as ordinate) could be satisfactorily linearized by weighted least-square regression to obtain standard curves for computerized routine assay of anti-*Candida* antibody. The computerized routine assay offers advantages as compared with other methods of quantitation. Results are printed out rapidly without need of further processing, while changes in day-to-day counts due to isotope decay and errors arising from end-point dilutions in quantitative determinations, were obviated.

Antibodies to whole cytoplasmic and carbohydrate antigen, respectively, are present in all sera from both adults and children. The lowest concentration (0.01 MAN), found in the serum of a 5-month-old baby with immunodeficiency (total IgG 800 mg/l) was more than one order of magnitude higher than the minimum concentration detectable by the assay. The distributions of antibody levels to whole cytoplasmic (Q_a) and carbohydrate (Q_b) antigens, respectively, in the sera of candidiasis-free adults cover the range 0.05–4 MAN. The Poisson-like distribution of anti- Q_b -antibodies (Fig. 10b: median 0.80 MAN, 95%-limit 2.5 MAN) is similar to the distributions of IgG- and IgM-antibody to cell-wall antigen reported by Cobb and Parratt (1978), who also detected antibodies to cell wall antigen in all individuals, probably

due to simple colonization by *Candida albicans*. The antigen fraction Q_b contains a large amount of cell-wall mannan. Thus an increase of anti- Q_b antibodies with low levels of anti-cytoplasmic protein antibodies should indicate *Candida* overgrowth and superficial infection (Müller, 1976).

Anti- Q_a antibody levels covered a wider range and were higher in several candidiasis-free adults, but the majority had low levels of anti- Q_a antibody (Fig. 10a: median 0.69 MAN, 95%-limit 2.8 MAN). The low concentration peak probably reflects the presence of a small amount of cell-wall mannan in the cytoplasmic antigen preparation (Syverson and Buckley, 1977). The high concentration tail of the distribution curve (Fig. 10a) could be attributed to persistent antibodies due to a previous infection. An acute invasive infection can raise the anti- Q_a antibody level to 120 MAN and we have found a slow decrease after successful therapy (Brombach et al., 1979).

The assay described is highly sensitive, simple, rapid, acceptably reproducible and suitable for routine application, and lends itself particularly to automation. Its increased sensitivity is a distinct advantage over other methods. The sensitivity permits small changes of antibody level to be detected in suspected candidiasis. A rapid increase in concentration of specific antibodies within a few days indicates invasive candidiasis; a *Candida* infection may be reasonably excluded if antibody is constant or decreasing (Mauch et al., 1980). To detect infection with *C. albicans* or other *Candida* species, it is important to use whole antigen. However, its heterogeneity and low affinity limits assay precision and the differential diagnostic value of antibody measurement. Reliable discrimination between superficial and deep-tissue infections requires improved specificity of the antigen fraction used in the assay.

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